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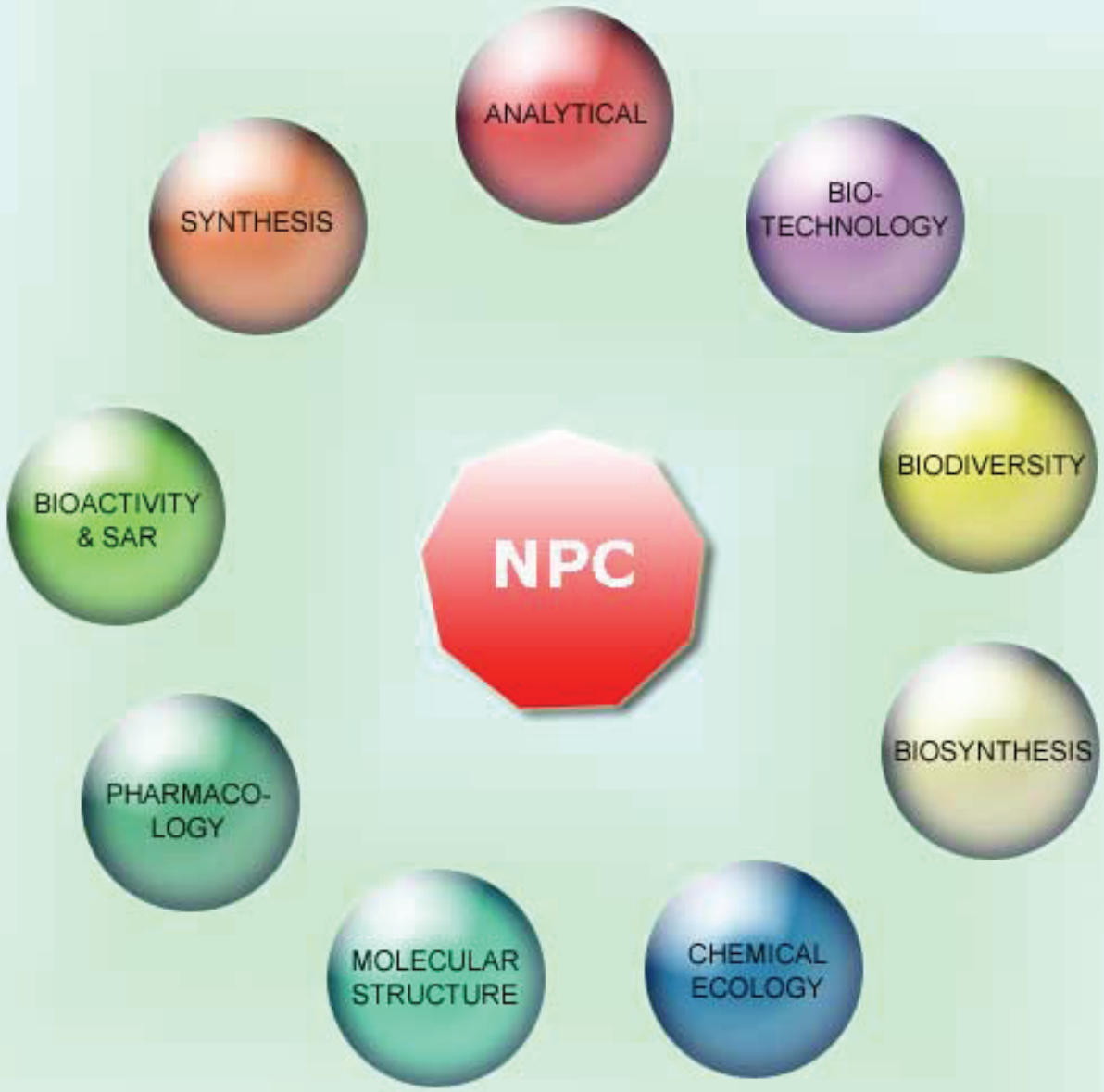

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Altered Antimicrobial and Anti-biofilm Forming Effect of Thyme Essential Oil due to Changes in Composition

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The antimicrobial and anti-biofilm forming effect of thyme (*Thymus vulgaris*) essential oil (TEO) with different compositions was evaluated. Normally the main component in this TEO is thymol, but in 2014 we found that the proportions of γ-terpinene and *p*-cymene (the precursors in thymol biosynthesis) increased and that of thymol decreased. This altered composition led to changes in the antimicrobial and anti-biofilm forming capacity of the essential oil depending also on the type of microorganisms. In the case of bacteria, minimal inhibitory (MIC) and minimal bactericidal (MBC) concentrations either decreased or increased. In the case of yeasts, minimal fungicidal concentrations (MFC) increased 4- and 8-fold for TEO containing *p*-cymene as the main component. On the contrary, MIC values decreased for all the tested moulds. Anti-biofilm forming activity of TEO containing *p*-cymene as its main component decreased in almost all cases and *P. fluorescens* biofilm forming capacity was even enhanced.

Keywords: Antimicrobial effect, Biofilms, Changed composition, Thyme, *Thymus vulgaris*, Essential oils.

The yield and quality of essential oils (EOs) and extracts depend on the edaphic, climatic conditions and the genotype of the plant. Geographical location, altitude, soil properties and irrigation influence the phytochemical composition and antioxidant activity of EOs. The chemical composition of EOs can be highly variable among cultivars, thus it is important to optimize both growing conditions and the genotype [1]. Furthermore, it is necessary to know the optimal harvesting time in order to provide high quality EOs [2].

EO yield is prominently influenced by the phenological stage (vegetative stage, full bloom, seed maturation) of the plant, but plant age [3, 4], leaf age and leaf positions [5] are also important. Seasonal changes [6], growth stages, origin of the herb [7], and the method of extraction can also influence yield, product quality, and quantitative and qualitative characteristics of EOs [8].

Usano-Alemany *et al.* studied EO yields and qualities of *Salvia lavandulifolia* and confirmed that plants in the vegetative and flowering stages have higher content of terpene hydrocarbons than those at the seed maturation stage, when the content of oxygenated derivatives increased remarkably [3].

The main components of *Thymus* EOs are thymol, *p*-cymene, γ-terpinene or carvacrol, and linalool, depending on the chemotype of the oil [9, 10, 11, 12].

The chemical compositions of EOs of *Thymus* species are also affected by environmental conditions (e.g., humidity, temperature, radiation, soil characteristics, moisture availability, fertility, electrical conductivity) and agronomic management practices. Higher elevation and colder temperature provided better growing conditions, and a higher accumulation of EO in thyme leaves [13].

Unsuitable environmental conditions may limit photosynthesis and change nutrient uptake in plants. Under stress (e.g., bad weather conditions, limited nutrients) they are more susceptible to pests and pathogens. During cold months with few hours of sunlight, decreased EO production was noticed [13].

Results of an experiment conducted in 2010 showed that changes in quality and quantity of EOs are species-specific under the same circumstances. The composition of EOs could change with the water supply. Using *Ocimum basilicum* L. as a test plant, the content of linalool decreased, but that of 1,8-cineol and tau-cadinol increased with increasing water scarcity. In the case of *Satureja hortensis*, the level of γ-terpinene increased, but that of carvacol decreased with increased water supply [14].

EOs are used in the food industry primarily as flavorings. They also have a potential food preservative ability because they are effective against food spoilage bacteria and mycotoxin producing fungi [15]. EOs could be ideal food additives instead of synthetic ones [16] as they are natural and mostly non-toxic to humans [17]. Most EOs have antibacterial activity against pathogens [18]. Besides this, studies show that they are also effective against bacterial biofilms [19, 20]. Biofilm formation is common among bacteria; in this form, they become more resistant to antimicrobials and sanitizing agents [21]. The antimicrobial activity of EOs is attributed to their components [22], which are generally classified into two groups of dissimilar biosynthetic origin. The main group contains terpenes and terpenoids, whereas the other one consists of aromatic compounds (phenylpropanoids) [23, 24].

The phenolic components are chiefly responsible for the antimicrobial effect as membrane permeabilizers. Gram-positive microorganisms are generally more sensitive to EOs than Gram-negative ones [12, 20, 25].

Table 1: Chemical composition (%) of the thyme essential oils tested.

Compound	Chemical composition (%)			
	2013		2014	
	Retention time (min)	Component proportion (%)	Retention time (min)	Component proportion (%)
1,8-Cineol	8.0-8.1	9.8	-	-
3-Carene	-	-	7.3	0.5
Borneol	11.0	1.0	10.7	0.5
Camphene	6.3	0.9	6.2	4.9
Carvacrol	13.3	3.2	13.2	3.1
Caryophyllene oxide	-	-	17.7	0.2
Isoborneol	-	-	10.6	0.4
Linalool	9.5	5.1	9.3	5.3
Linalyl acetate	-	-	12.4	0.3
Myrcene	-	-	6.9	4.0
Nerol	-	-	12.6	0.1
Neryl acetate			14.4	0.3
p-Cymene	7.9	22.1	7.8	30.1
Terpinen-4-ol	11.5	0.6	10.9	0.9
Terpinolene			9.0	0.9
Thymol	13.2	46.3	13.1	23.2
α-Humelene	-	-	15.8	0.2
α-Pinene	5.9	0.9	5.8	3.2
α-Terpinene	7.7	2.2	7.6	5.9
α-Terpineol	-	-	11.4	0.6
β-Caryophyllene	15.4	2.5	15.2	2.5
β-Pinene	6.9	1.4	6.7	0.8
γ-Terpinene	8.6	0.3	8.5	10.0
Terpene hydrocarbons		30.3		62.1
Terpenoids		66.0		34.9
Total		96.3		97.0

Our group has been working with EOs for years. We worked with a thymol chemotype of *Thymus vulgaris* L. EO in which thymol always formed the highest proportion of the main components. In 2014, after a rainy summer, the composition of the TEO changed dramatically; this initiated us to examine if changes in the composition of the essential oil had had an influence on its antimicrobial efficiency.

Table 1 presents the chemical compositions of *T. vulgaris* EO from different years. Chromatographic analysis showed that the major component of thyme EO in 2013 was thymol and in 2014 it was its precursor, *p*-cymene. 1-8 Cineol was detected only in 2013, while the content of γ-terpinene was much higher in the following year.

In most cases, the MIC and MBC values changed in 2014, except for *Bacillus subtilis* for which no changes were observed. MIC and MBC values for *Staphylococcus aureus* and *Pseudomonas fluorescens* increased compared with values in 2013 (Table 2).

Table 2: MIC and MBC values of TEO with original and altered composition against bacteria (mg/mL).

Bacteria	MIC (mg/mL)		MBC (mg/mL)	
	2013	2014	2013	2014
E. coli	1.6	1.6	1.6	32.0
P. putida	20.0	8.0	>100	8.0
P. fluorescens	1.6	2.0	1.6	3.2
B. cereus	1.6	0.8	3.2	1.6
B. subtilis	0.8	0.8	1.6	1.6
S. aureus	0.8	1.6	1.6	3.2
MRSA	3.2	1.6	12.5	3.2
L. monocytogenes	1.6	3.2	6.3	6.3

The MBC of *Escherichia coli* increased twenty times, while that of *Pseudomonas putida* decreased by about one tenth compared with 2013 values. MIC and MBC values of MRSA and *B. cereus* var. *mycoides* decreased moderately.

Sessile cells in biofilms of most investigated bacteria showed different results compared with planctonic cells. Almost in all cases, thyme EO from 2013 was more effective than that from 2014 (Figure 1). For *B. cereus* var. *mycoides* there was no significant difference between the two *Thyme* EO preparations, but in the case

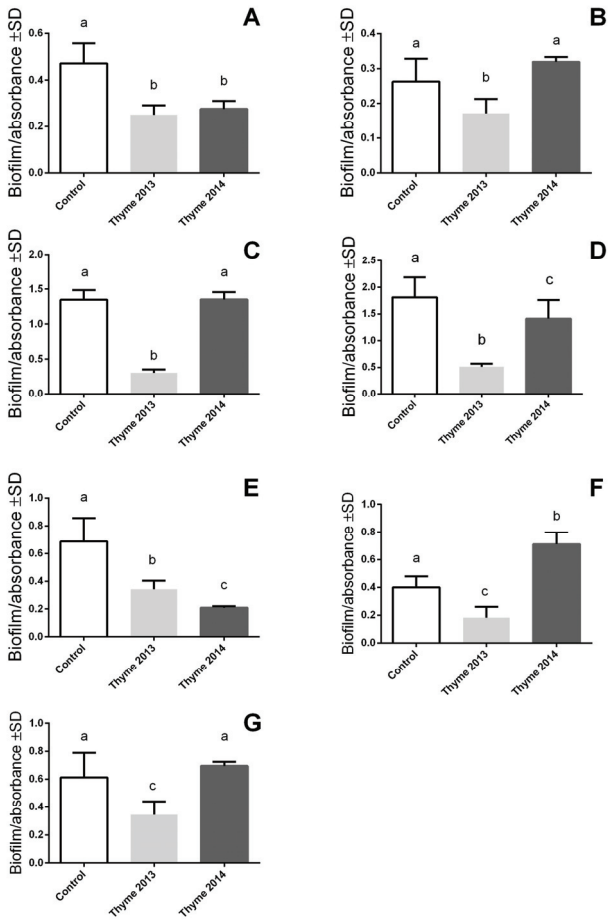

Figure 1: Anti-biofilm forming effect of TEO from 2013 and 2014. The oil was used in MIC/2 concentration. A: *B. cereus*; B: *B. subtilis*; C: *L. monocytogenes*; D: *P. putida*; E: *E. coli*; F: *P. fluorescens*; G: *P. anomala*.

of *E. coli* the EO containing more *p*-cymene (2014) was more effective. *P. fluorescens* biofilms were enhanced by the EO, which is in agreement with the findings of Sandasi *et al.* where monoterpenes enhanced biofilm formation [26]. It seems that by the accumulation of *p*-cymene and the reduction of thymol the biofilm formation was stimulated instead of inhibited.

In the case of yeasts, the MFC values increased 4-, and 8-fold from 2013 to 2014. On the contrary, MIC values decreased for all the tested moulds (Table 3).

Table 3: MFC values of thyme EO with original and altered composition tested *in vitro* against yeasts and moulds (mg/mL).

Fungi	Year	
	2013	2014
Candida albicans	0.78	3.12
Candida parapsilosis	0.19	1.56
Pichia anomala	1.56	1.56
Aspergillus fumigatus	0.39	<0.19
Aspergillus terreus	0.78	0.39
Fusarium solani	0.78	0.39
Rhizopus microsporus	0.19	<0.19

In all cases, thymol alone showed better antimicrobial activity than *p*-cymene. Mixing the two components resulted in mainly a stepwise increase in the MIC and MBC values (Table 4). For staphylococci, the first increase occurred at the ratio of thymol: *p*-cymene of 4:1, while for the other bacteria the ratio was either 1:1 or 3:2. MIC and MBC values of the compounds either alone or in combination were in the same range as those of the whole EO.

Table 4: MIC/MBC values (mg/mL) after treatment with thymol and *p*-cymene in different proportions.

	E. coli		*S. aureus*		MRSA		*L. monocytogenes*		*B. subtilis*		*B. cereus*	
Thymol: *p*-Cymene (v:v)	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
100:0	0.20	0.40	0.10	0.20	0.10	0.20	0.40	0.80	<0.05	0.05	0.40	0.80
80:20	0.20	0.40	0.20	0.40	0.20	0.40	0.40	0.80	<0.05	0.05	0.40	0.80
60:40	0.20	0.80	0.20	0.40	0.20	0.40	0.40	0.80	0.05	0.10	0.40	0.80
50:50	0.20	0.80	0.20	0.40	0.20	0.40	0.80	1.60	0.20	0.40	0.80	0.80
40:60	0.40	0.80	0.20	0.40	0.20	0.40	0.80	1.60	0.20	0.40	0.80	1.60
20:80	0.80	1.60	0.20	0.40	0.40	0.80	1.60	3.20	0.20	0.40	0.80	1.60
0:100	1.60	3.20	0.80	1.60	0.80	1.60	3.20	>25.00	0.80	1.60	0.80	1.60

Several authors reported the importance of different environmental factors to the EO compositions of plants [2, 14, 27]. Drought stress, but also too much rain and little sunshine, can lead to changes in the biochemical pathways of the synthesis of EO compounds. In Hungary (and in the whole of Central Europe), summer showed a lot of extremities in 2013 and 2014. Temperature increased from 2013 to 2014, but in contrast to the global trend, in Hungary 2014 was rainier than 2013.

It seems that this unusual climate of 2014 affected the EO biosynthesis of *Thymus* plants. Thymol is synthesized from *p*-cymene, which in turn is derived from γ-terpinene [28]. In the EO from 2014 the precursors: *p*-cymene and γ-terpinene were found in large amounts while thymol was reduced to about a half (Table 1).

This altered composition led to changes in the antimicrobial and anti-biofilm forming activity of the oil. The data for the antimicrobial effect obtained with *p*-cymene and thymol alone and in combinations (Table 4) showed that thymol alone had better antimicrobial activity than *p*-cymene either alone or in any combination of the two. Dorman and Deans also described the relative inefficiency of *p*-cymene compared with that of thymol [22]. However, the EO with decreased thymol and increased *p*-cymene and γ-terpinene content (year 2014) showed, in certain cases, higher antimicrobial activity than the EO from 2013. This indicated that not only the main ingredients had a crucial role in the antimicrobial activity, but the individual sensitivity of the used strains was also very important.

In 2014, the proportion of terpenes increased at the expense of terpenoids. It is generally assumed that terpenoids have better antimicrobial effect than terpenes [22, 23], but synergistic interactions among monoterpene hydrocarbons and terpenoids cannot be excluded.

This study investigated the antimicrobial and anti-biofilm forming effects of thyme essential oils from plants harvested in two consecutive years. The major component of the TEO in 2013 was thymol and in 2014 (with hot and rainy weather), its precursor, *p*-cymene. The altered composition led to remarkable changes in the antimicrobial and anti-biofilm forming capacity of TEO. Thymol alone had better antimicrobial effect than *p*-cymene alone or in any combination of the two, but the minor components in the whole TEO could modulate this effect.

An important conclusion of our finding is that, as plant EO compositions cannot be standardized, MIC values need to be established for every new batch of EO.

Experimental

Test organisms: Selected bacteria and fungi from the Szeged Microbiology Collection (SZMC; WDCM987) and American Type Culture Collection (ATCC; WDCM1) were investigated. The investigated bacteria were: *Bacillus cereus* var. *mycoides* SZMC 0042, *B. subtilis* SZMC 0209, *Escherichia coli* SZMC 0582, *Listeria monocytogenes* SZMC 21307, *Pseudomonas putida* SZMC 291T, *P. fluorescens* SZMC 16106, *Staphylococcus aureus* ATCC 43300 (methicillin resistant, (MRSA) and ATCC 25923 (antibiotic sensitive).

S. aureus and *E. coli* strains were grown in LB broth: 10 g NaCl (VWR, Belgium), 10 g bacto peptone (Oxoid, England), 5 g yeast extract (HiMedia, India) in 1000 mL. *B. cereus, B. subtilis,* and *P. putida* were cultivated in TGE broth: 10 g glucose (VWR, Belgium), 5 g bacto peptone, 2.5 g yeast extract, in 1000 mL. TSB was used for *L. monocytogenes*: 17 g casein peptone (Merck, Germany), 3 g soy peptone (Oxoid, England), 2.5 g glucose, 5 g NaCl, 2.5 g K_2HPO_4 (Reanal, Hungary), in 1000 mL. *E. coli, S. aureus*, and *L. monocytogenes* were incubated at 37°C, *P. putida* at 25°C, and bacilli at 30°C for 18-20 h to reach early stationary growth phase.

The tested fungal species were: *Aspergillus fumigatus* SZMC 2394, *A. terreus* SZMC 2414, *Fusarium solani* SZMC 11412, *Rhizopus microsporus* SZMC 13644, *Candida albicans* SZMC 1363, *C. parapsilosis* SZMC 1408, and *Pichia anomala* SZMC 8061. For the cultivation of yeasts and moulds malt extract agar was used (0.4% malt extract, 0.1% yeast, 1% glucose, and 2% agar).

Essential oils and components: Thyme *(Thymus vulgaris)* essential oils produced in 2013 and 2014 were purchased from Aromax Natural Products Ltd. (Budapest, Hungary). EOs were prepared by steam distillation of aerial parts of the plants.

Thymol and *p*-cymene were purchased from Sigma Aldrich (Budapest, Hungary).

Identification of essential oil compounds

Solid phase microextraction (SPME) conditions: Essential oil (0.5 mL) was put into vials (20 mL headspace) and sealed with a silicon/PTFE septum prior to SPME-GC/MS analysis. Sample preparation using a static headspace solid phase microextraction (sHS-SPME) technique was carried out with a CTC Combi PAL (CTC Analytics AG, Zwingen, Switzerland) automatic multipurpose sampler using a 65 μM StableFlex polydimethylsiloxane/divinyl benzene (PDMS/DVB) SPME fiber (Supelco, Bellefonte, PA, USA). After an incubation period of 5 min at 100ºC, extraction was performed by exposing the fiber to the headspace of a 20 mL vial containing the plant material for 20 min at 100ºC. The fiber was then immediately transferred to the injector port of the GC/MS, and desorbed at 250ºC for 1 min. The SPME fiber was cleaned and conditioned in a Fibre Bakeout Station in a pure nitrogen atmosphere at 250ºC for 15 min.

GC-MS conditions: The analyses were carried out with an Agilent 6890N/5973N GC-MSD (Santa Clara, CA, USA) system equipped with an Agilent SLB-5MS capillary column (30 m × 250 μm × 0.25 μm). The GC oven temperature was programmed to increase from 60ºC (3 min isothermal) – 250ºC at 8ºC/min (1 min isothermal). High purity helium (6.0) was used as carrier gas at 1.0 mL/min

(37 cm/s) in constant flow mode. The injector temperature was 250°C and the split ratio was 1:50. The mass selective detector was equipped with a quadrupole mass analyzer and was operated in electron ionization mode at 70 eV in full scan mode (41–500 amu at 3.2 scan/s). The data were evaluated using MSD ChemStation D.02.00.275 software (Agilent). The identification of the compounds was carried out by comparing retention times and recorded spectra with the data of authentic standards, and the NIST 2.0 library was also consulted.

Determination of minimum inhibitory and minimum bactericide concentrations: For minimum inhibitory concentration (MIC) determination, the broth microdilution method was used. In 96-well microtiter plates (Costar, USA) 100 µL cell suspensions (10^5 CFU/ml) were mixed with 100 µL EO solution containing 0.1% TWEEN 40 in the concentration range between 0.1-100 mg/mL. Negative controls were EOs in sterile medium with TWEEN 40, and positive controls were inoculated media without EO. After 18-24 h incubation at the appropriate temperatures, growth of the bacteria was determined after staining with 20 µL resazurin (Cell Titer-Blue® Reagent, Promega) indicator. Color was checked after 1 h. Oxido-reductase enzymes of living cells reduce the blue colored resazurin to the pink colored resorufin [29]. MIC was determined as the lowest concentration where the blue colour did not change. Six replicates were prepared for each EO concentration.

Minimum bactericide concentration (MBC) was detected by the tracking plate method [30] transferring 10 µL from the microplate wells corresponding to MIC and higher concentrations on suitable medium. After 24 h incubation at appropriate temperature, the appearance of bacterial colonies was checked. MBC was defined as the EO concentration where no colony growth was observed.

Biofilm formation and treatment: Microtiter plates (96-well) were inoculated with 200 µL of 24-h-old liquid culture (10^8cfu/mL). Following 4 h of cell adhesion, the supernatant was removed, and the plates were rinsed with physiological saline. Fresh medium (200 µL) containing TEO was added in MIC/2 concentration to each well, and the plates were further incubated for 24 h. Positive controls contained the inoculated growth medium without any TEO, and negative controls contained TEO in growth medium. Biofilms were visualized after crystal violet staining, as described by Kerekes *et al.* [19]. Experiments were repeated at least 2 times with 6 parallel measurements.

Determination of minimal fungicidal concentration: Minimal fungicidal concentration (MFC) values were determined by macrodilution for yeasts, and by food poisoning method for moulds, as described in the literature [31].

For yeasts, test tubes contained 1 mL malt extract medium and 1 mL EO solution to obtain final concentrations of 25 - 0.2 mg/mL in a twofold dilution range. Tween 40 (1%) was used for the solubilization of EOs. Test tubes were inoculated with 0.1 mL of 24-h-old yeast suspension (10^5 cfu/mL). After 24-h incubation, the growth was checked by the tracking plate method, as described earlier. Plates were incubated at 30°C for 24 h, and then the number of colonies was evaluated. MFC was defined as the EO concentration where no colony growth was observed.

Spore suspensions (10 µL, 10^5 cfu/mL) from 72-h-old mould cultures were dropped onto plates containing 15 mL medium and 0.1% Tween-40 supplemented with EOs in the concentration range of 25-0.2 mg/mL. Petri dishes were sealed with parafilm and incubated at 25°C for 7 days. Control plates (without EOs) were inoculated by the same procedure. After 7 days of incubation the MFC was defined as the minimum EO concentration at which no visible growth was observed. For each concentration 3 parallel measurements were carried out.

Antimicrobial effect and interaction of thymol and p-cymene: The antimicrobial effect of thymol and its precursor *p*-cymene was investigated alone and in combinations. The tested ratios were (thymol: *p*-cymene): 4:1, 3:2, 1:1, 2:3, and 1:4. Stock solutions (100 mg/mL) were prepared in 96% ethanol. Antimicrobial effect was determined by the broth microdilution method. Wells containing the components alone or in combination mixed with the appropriate media were inoculated with bacteria (10^5 cfu/mL) and incubated for 24 h. After that, absorbance was measured at 595 nm (SPECTROstar microplate reader, BMG Labtech). The concentration at which absorbance decreased below 10% of the positive control was considered as MIC.

Statistical analysis: Data were analyzed using one-way ANOVA (GraphPad Prism 6.0). Means were compared by the Tukey's test and level of significance was $p<0.05$.

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